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Functional study of hyperpolarization activated channel (I_h) in *Drosophila* behavior

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Hyperpolarization-activated, cyclic nucleotide-gated and cation-nonselective ion channels (I_h channels, or HCN channels) are known to play important roles in mammals. Their physiological functions in invertebrate remain largely unclear. Here, we report our studies with I_h channel in *Drosophila melanogaster*. *Drosophila* I_h channel mutants are found with several defects by behavioral analyses. Their lifespan is reduced, and their chemical sensitivity is shifted. In addition, their length of sleep at light-dark condition is mildly reduced. We generated transgenic flies of I_h promoter-driven Gal4 and examined its expression pattern in both larvae and adult flies. Our results suggest that I_h channel may play diverse roles in *Drosophila* and provide a basis to further expand our understanding of *Drosophila* I_h channel function *in vivo*.

I_h channel, *Drosophila melanogaster*, HCN, behavior

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In mammals, hyperpolarization-activated, and cyclic nucleotide-gated cation channels (HCN or I_h channel) play diverse functional roles, including maintenance of pacemaker activity for neurons and heart cells, regulation of membrane excitability, dendritic integration, and neuronal plasticity [1–3]. Knock-out of individual I_h channel genes in mice leads to impaired learning and memory ($HCN1^{-/-}$) [4,5], absence epilepsy and sinus dysrhythmia ($HCN2^{-/-}$) [6], and heart contraction defect ($HCN4^{-/-}$) [7].

The invertebrate counterpart of I_h channels is less functionally studied. In *Drosophila melanogaster,* there is only one I_h channel gene [8,9]. *Drosophila* I_h was reported to be strongly expressed in the compound eyes, the second segments and third segments of the antennae [9]. When *in vitro* expressed in HEK293 cells, *Drosophila* I_h channel was demonstrated to mediate inward current upon hyperpolarization. As is expected, its activation kinetics shifts with cellular cAMP level [10]. However, the *in vivo* function of *Drosophila* I_h channel remains unknown. In this study, we carried out behavioral analyses of fly I_h channel mutants and found several defects for I_h channel mutants. We also generated transgenic lines of I_h promoter-driven Gal4 and characterized its expression pattern.

1 Materials and methods

1.1 Fly stocks

I_h mutants (I_h^{f03355} and I_h^{f01485}) were ordered from Havard Exelixis stock centre. Df(2R)Exel7131 was ordered from Bloomington Stock Centre. An excision line was generated from I_h^{f03355} by crossing with a line carrying piggyBac transposase.

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To generate I_h promoter-driven Gal4, one 3.2 kb DNA fragment was PCR-amplified from Canton-s genomic DNA using primers (5′-attagcggccgcCTATTGGAGGAGAAG-GAGCAGT-3′ and 5′-attagtttaaacGAAATGCGGGATAA-CGGACG-3′), and then inserted into pCasper-AUG-Gal4 plasmid vector. Transgenic I_h -Gal4 lines were generated by P element-mediated transformation.

1.2 Immunochemistry

"UAS-mCD8-GFP; I_h-Gal4(Line76)" was used to examine the expression pattern of I_h. Rabbit anti-GFP (1:1000, Invitrogen) and nc-82 (1:100, DHSB) were used as primary antibody. Alexa 488 anti-rabbit (1:100, Invitrogen) and Alexa 556 anti-mouse (1:100, Invitrogen) were used as the secondary antibody. Images were acquired by using a Zeiss LSM confocal microscope system.

1.3 Lifespan analysis

I_h mutant (*f03355* homozygote) and its genetic background control w^{1118} were analyzed for their lifespan. Newly eclosed male flies were simultaneously collected and reared at a density of ~20 flies per vial. 179 flies for *f03355* mutant and 159 flies for w^{1118} control were tested. Fly vials were laid horizontally in an artificial climate incubator (25°C and 70% RH, with 12L:12D light cycle). The number of alive flies was recorded, when they were transferred every two days.

1.4 Proboscis extension (PER) assay

PER assays were performed out as described [11]. Newly eclosed flies were fed on regular food for 24 h, and then starved with water for 24 h. Flies were anaesthetized briefly with CO_2 and immobilized on slides with myristic acid. Flies were then allowed to recover in a humid chamber for 2 h. After that, flies were given water to drink three times. Finally, flies were tested for each taste stimulus three times. Bitter substances were mixed with 100 mmol/L sucrose to test. For each genotype, three batches of 20–35 flies were tested on different days as independent replicates.

1.5 Sleep analyses

Drosophila activity monitor system was used to analyze sleep phenotype. Flies were entrained with 12L/12D (LD condition) for 3 d and then reared with constant darkness (DD condition) for another 3 d. The activity of each fly was monitored per minute. Any fly with no activity in 5 min was defined as sleep. The daily sleep time and bout number for each fly were recorded and used for further analyses.

1.6 Wing phenotype

About 200 "UAS-NaChBac;I_h-*Gal4*" flies were examined for their wing morphology. The wing phenotype was analyzed as described [13], including normally extended wings (EW), partially extended wings (PEW), or un-extended wings (UW).

2 Results

2.1 Characterization of I_h mutants in *Drosophila melanogaster*

To study the *in vivo* function of I_h in *Drosophila melanogaster* by mutant analyses, three PiggyBac insertion lines (*e01599*, *f03355*, and *f01485*, see Figure 1A) were obtained [12]. The results of RT-PCR experiments confirmed that I_h transcription was indeed disrupted in *f03355* and *f01485* (Figure 1B). PiggyBac insertion in *f03355* was found to split I_h RNA into two halves, which is supposed to truncate I_h channel between S2 and S3 trans-membrane segment. As for *f01485*, an extra 373 nucleotide from piggybac was spliced into I_h transcripts, which results in frameshift of I_h channel after S4 trans-membrane segment. Since the integrity of six trans-membrane segments is absolutely required for I_h channel activity, we speculated that both *f03355* and *f01485* were null mutation lines for I_h.

2.2 Survival curves of w^{1118} and I_h mutant *f03355*

No general developmental defects were observed in *f03355* and *f01485* homozygote. They were both viable and fertile. At young age, they did not obviously differ from wild-type. However, at old age, they were strongly uncoordinated in movement and easier to fall down, with their bodies quivering frequently. *f03355* mutant was found with a shortened lifespan. For example, on day 51, all analyzed *f03355* mutant flies were already dead, while 45% of w^{1118} control flies were still alive (Figure 2). This demonstrated that I_h channel indeed plays certain important roles in *Drosophila*.

2.3 Taste behaviors are changed in I_h mutants

To test whether I_h channel is involved in sensory systems, proboscis extension assays were carried out. As shown in Figure 3, *f03355* homozygote flies displayed an enhanced proboscis extension response (PER) to sugar stimuli, compared with w^{1118} control (Figure 3A and B). The enhanced PER to 40 mmol/L glucose by I_h mutant *f03355* was recessive and was not complemented by a deficiency line covering the I_h locus. Such phenotype was recovered when the piggybac insertion in *f03355* was excised. Moreover, the enhanced PER phenotype was also observed in another allele, I_h *f01485* mutant (Figure 3B). As is shown in Figure 3C, *f03355* mutants also showed higher PER than control w^{1118} flies, when checked with bitter substance, such as caffeine and berberine. Thus, I_h mutants were found with a general increased response to appetitive substance.

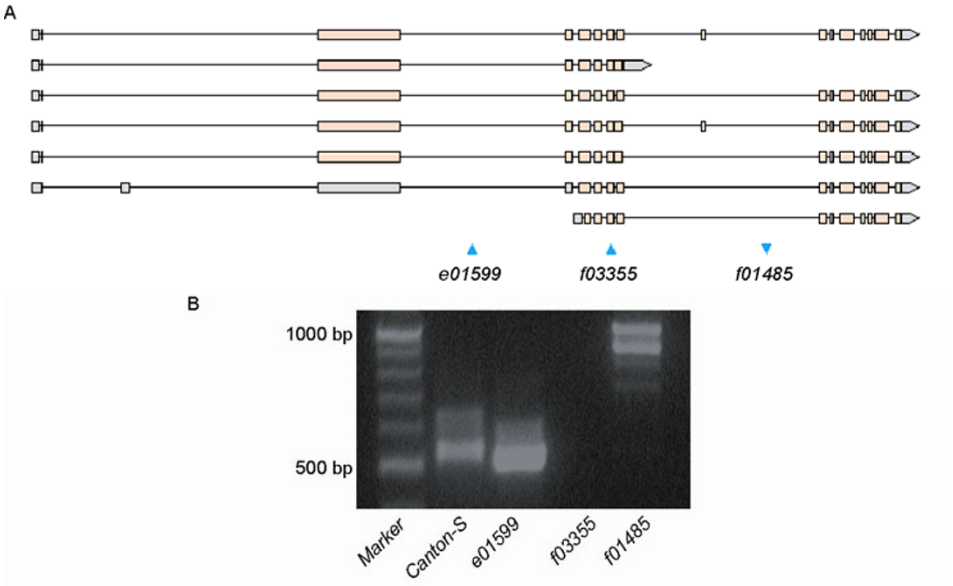

Figure 1 Characterization of I_h mutants in *Drosophila melanogaster*. A, Schematic representation of the structure of I_h gene and its mutants (*e01599*, *f03355 and f01485*). The annotated transcription for *Drosophila* I_h gene, and PiggyBac insertion sites of I_h mutants (*e01599*, *f03355* and *f01485*) are indicated; B, The expression of I_h gene in wild-type control flies (Canton-s) and three I_h mutants (*e01599, f03355* and *f01485*) were examined by RT-PCR using I_h-specific primers.

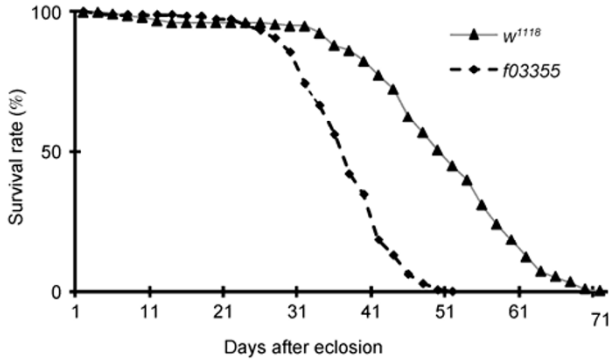

Figure 2 Survival curves of wild-type (w^{1118}) and I_h mutant (*f03355*) flies are shown. There is a significant difference between the two groups ($P<$ 0.001, log rank test).

2.4 Reduction in daily sleep time in I_h mutants

To test whether I_h channel is also involved in complicated behaviors such as sleep, I_h mutant *f03355* was examined using *Drosophila* activity monitor system. At LD condition, both gender of *f03355* mutant were discovered with a reduction in daily sleep time. For *f03355* male flies, sleep bout number was increased (Table 1). At DD condition, only sleep bout number for female *f03355* flies was with an increase in bout number (Table 2).

2.5 Expression pattern of I_h-*Gal4 flies*

The above results indicated that I_h channel probably plays several different roles in *Drosophila*. To further understand its physiological function and the underlying mechanism, we examined I_h gene expression in more detail. As a result, I_h promoter-fusion Gal4 lines were generated and their expression patterns were revealed by immunostaining (Figure 4). I_h-*Gal4* specifically labeled lateral chordotonal neurons of the peripheral nervous system in third-instar larvae (Figure 4A). For adult brain, peripheral chordotonal projection to antennal mechanosensory and motor centre can also be visualized (Figure 4A). Interestingly, I_h-*Gal4* also labeled several subesophageal ganglia neurons, which are the potential target for I_h to modulate taste responses. Moreover, I_h-*Gal4* also labeled some other neurons in the central brain, including several local interneurons in the antennal lobes (Figure 4B).

2.6 Hyperexcitation of I_h-*Gal4* positive neurons results in wing extension defects

In an effort to reveal the function of those neurons labeled by I_h-*Gal4* line, the bacterial sodium channel (NaChBac) was over-expressed in such neurons to elevate the level of their neuronal activity [13–15]. Such manipulation resulted in wing extension defect. About 48% of those flies were shown with unexpanded wings (UEW), and 9% of flies partially extended their wings (PEW) (Figure 5).

3 Discussion

In this study, we characterized and analyzed *Drosophila* I_h mutants. For *Drosophila melanogaster*, I_h channel seems to exert rather diverse functions, from chemosensory modula-

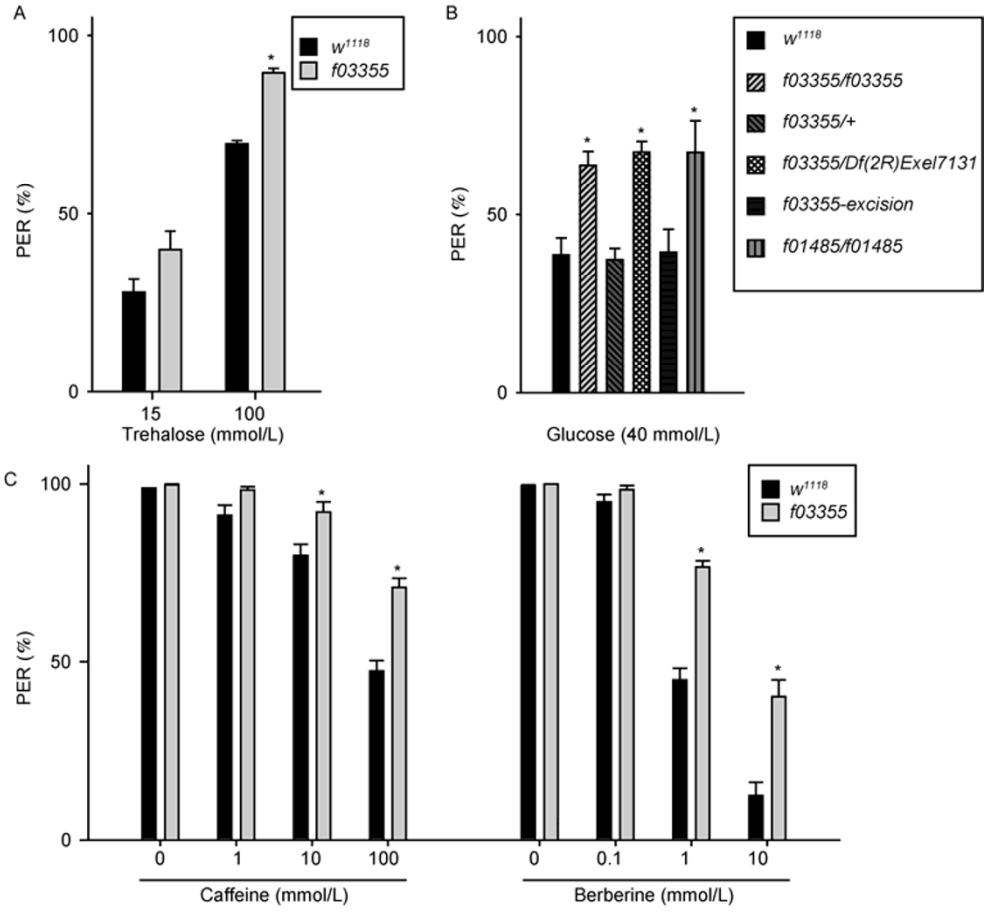

Figure 3 Taste responses in I_h mutant flies as shown by proboscis extension responses are shifted in I_h mutants (*, P <0.05, t-test). The respective stimuli in each panel are as follows: A, trehalose: 15 or 100 mmol/L; B, glucose: 40 mmol/L; C, caffeine: 1, 10 or 100 mmol/L, and berberine: 0.1, 1 or 10 mmol/L.

Table 1 Sleep phenotype for I_h mutant (*f03355*) at light-dark condition

Genotype	Sleep time (min)	Bout number
w^{1118} ♀ (n=27)	936±22	42±1.8
f03355 ♀ (n=28)	867±22 (P<0.05)	41±1.7
w^{1118} ♂ (n=25)	1063±22	32±1.5
f03355 ♂ (n=28)	940±31 (P<0.05)	48±1.7 (P<0.05)

Table 2 Sleep phenotype for I_h mutant (*f03355*) at constant dark condition

Genotype	Sleep time (min)	Bout number
w^{1118} ♀ (n=27)	910±26	53±2.2
f03355 ♀ (n=28)	942±32	60±2.0 (P<0.05)
w^{1118} ♂ (n=25)	920±34	50±2.3
f03355 ♂ (n=28)	892±34	53±1.3

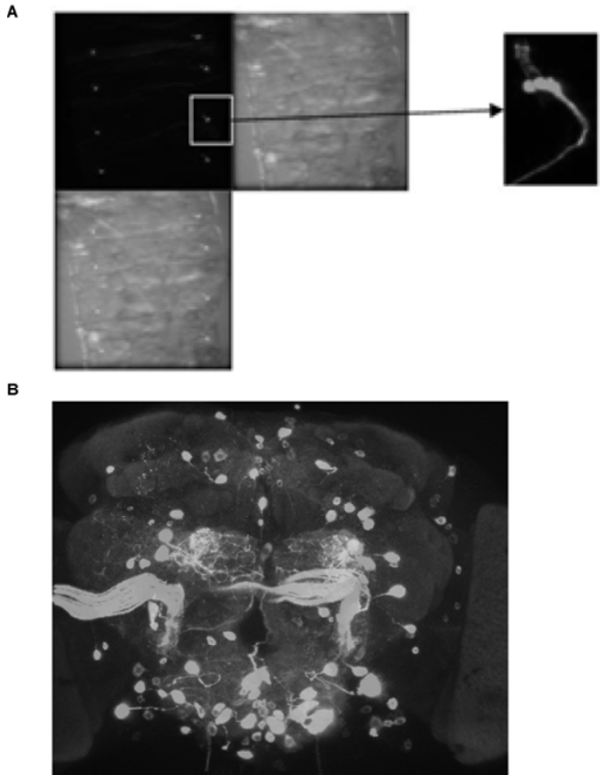

Figure 4 Expression pattern of I_h-*Gal4* flies. The reporter GFP gene was expressed under the control of I_h promoter-*Gal4,* and visualized by immunostaining. A, For larvae cuticle, I_h-*Gal4* labels lch5 chordotonal neurons. B, For adult brain, I_h-*Gal4* are expressed in several neurons within subesophageal ganglion, antennal lobe and other regions. Projection from Johnston's organ to antennal mechanosensory and motor centre can also be visualized.

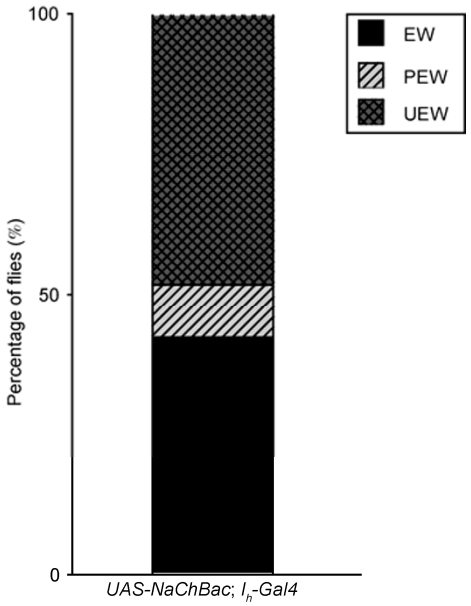

Figure 5 Hyperexcitation of I_h-*Gal4* positive neurons results in wing extension defects. Percentage of each population with different wing morphology (EW, wings normally extended; PEW, wings partially extended; UEW, wings not extended) is shown.

tion to sleep regulation. We found that the lifespan of *Drosophila* I_h channel mutants was reduced, and their sensitivity to bitter and sweet substance was changed. Their sleep at light-dark condition was mildly reduced. Taken together, we suggest that I_h channel may be involved in the regulation of multiple *Drosophila* behaviors.

The generation of I_h-*Gal4* and analyzing its pattern will be informative to tissue specifically dissect I_h functional roles. Our work provides a basis for further studying I_h channel physiologically, by combining the available resource of different genetic tools. It will also be very promising to test I_h interaction with other mutants in *Drosophila*. For example, is the enhanced appetitive taste phenotype in I_h mutant is dependent on *forage* gene background? Because it is known that rover and sitter alleles for *forage* were also shown with different PER responses [16], and *forage* encodes PKG of *Drosophila*, which can potentially modulate I_h channel. Such genetic interaction studies can provide view ideas for the field of I_h channel. Our studies may provide a basis to further expand our understanding of *Drosophila* I_h channel function *in vivo*.

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